

Efficient Retrovirus Transduction of Mouse Pluripotent Hematopoietic Stem Cells Mobilized Into the Peripheral Blood by Treatment With Granulocyte Colony-Stimulating Factor and Stem Cell Factor

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Cytokine-mobilized peripheral blood cells have been shown to participate in hematopoietic recovery after bone marrow (BM) transplantation, and are proposed to be useful targets for retrovirus-mediated gene transfer protocols. We treated mice with granulocyte colony-stimulating factor (G-CSF) and stem cell factor (SCF) to mobilize hematopoietic progenitor cells into the peripheral blood. These cells were analyzed for the number and frequency of pluripotent hematopoietic stem cells (PHSC). We found that splenectomized animals treated for 5 days with G-CSF and SCF showed a threefold increase in the absolute number of PHSC over normal mice. The number of peripheral-blood PHSC increased 250-fold from 29 per untreated mouse to 7,200 in peripheral-blood PHSC in splenectomized animals treated for 5 days with G-

CSF and SCF. Peripheral blood PHSC mobilized by treatment with G-CSF and SCF were analyzed for their ability to be transduced by retroviral vectors. Peripheral-blood PHSC from splenectomized animals G-CSF and SCF were transduced with a recombinant retrovirus containing the human *MDR-1* gene. The frequency of gene transfer into peripheral blood PHSC from animals treated for 5 and 7 days was two-fold and threefold higher than gene transfer into PHSC from the BM of 5-fluorouracil-treated mice ($P < .01$). We conclude that peripheral blood stem cells mobilized by treatment with G-CSF and SCF are excellent targets for retrovirus-mediated gene transfer.

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IN NORMAL individuals the number and frequency of hematopoietic progenitor cells in the peripheral circulation is only a small fraction of the number and frequency of similar progenitor cells in the bone marrow (BM).¹ The infusion of hematopoietic growth factors or cytokines into patients has been shown to result in an increase in both the number and frequency of hematopoietic progenitor cells (CFU-C, CFU-mix, burst-forming unit-erythroid [BFU-E], etc) in the peripheral blood.^{2,3} These mobilized hematopoietic progenitor cells have been collected and used to provide support for autologous BM transplantation (BMT) in patients undergoing high-dose chemotherapy for breast or ovarian cancer.⁴⁻⁶ The infusion of mobilized hematopoietic progenitor cells from peripheral blood together with BM cells has been shown to reduce the period of neutropenia after transplantation.⁷ In addition, hematopoietic engraftment has been shown after the transplantation of mobilized peripheral blood cells alone.⁸ The ability of peripheral blood cells to participate in hematopoietic recovery has lead to the hypothesis that pluripotent hematopoietic stem cells (PHSC) are among the cells mobilized into the peripheral blood.^{7,9}

Animal models have been used to confirm this hypothesis. Peripheral blood cells collected after treatment with granulocyte colony-stimulating factor (G-CSF) or stem cell factor (SCF) have been shown to completely repopulate lethally irradiated mice and baboons.¹⁰⁻¹² We have previously treated mice with SCF. Compared with untreated mice, SCF-treated animals have threefold more PHSC/animal, with 10- and 14-

fold increases in the number of peripheral blood and spleen PHSC, respectively.¹³

In vitro studies have shown that mobilized peripheral blood progenitor cells are efficiently transduced by recombinant retroviruses.^{14,15} In a study by Cassel et al¹⁴ the efficiency of gene transfer into CFU-C mobilized into the peripheral blood by a combination of cytokines and cytotoxic drugs was compared with the efficiency of gene transfer into BM CFU-C. The results showed clearly that the mobilized peripheral blood progenitor cells were more efficiently transduced, leading the investigators to propose that mobilized peripheral blood hematopoietic cells might be an alternative to BM cells for gene-therapy protocols.^{13,14}

In our previous study we showed that the efficiency of retrovirus-mediated gene transfer into SCF-mobilized peripheral blood and spleen PHSC was less efficient than gene transfer into PHSC in the BM of 5-fluorouracil (5-FU)-treated mice.¹³ Bridell et al¹⁶ and others have recently shown that mice treated with both G-CSF and SCF have greater numbers of hematopoietic progenitor cells in the peripheral blood than mice treated with either cytokine alone. The results were particularly pronounced in splenectomized animals.¹⁶⁻¹⁸

In this study we evaluate splenectomized mice treated with the combination of G-CSF and SCF for both gene transfer efficiency into peripheral blood PHSC and for the number and distribution of PHSC in the treated animals. Our results show that treatment with the combination of G-CSF and SCF causes a greater expansion of peripheral blood PHSC than treatment with either cytokine alone. In addition, the efficiency of gene transfer into peripheral blood PHSC collected after 5 or 7 days of treatment with G-CSF and SCF was greater than the efficiency of gene transfer into PHSC from the BM of 5-FU-treated mice.

MATERIALS AND METHODS

Mice. All mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Young adult (6 to 8 weeks) female, intact or splenectomized C57BL/6J mice were used as donors of peripheral blood or BM in all experiments. Experiments involving splenectomized mice were initiated at least 14 days postsplenectomy. Competitor marrow for the competitive repopulation assay was obtained from B6.C H-1^b/ByJ (H-2K^d) mice, which are congenic with C57BL/

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6J mice except for single allelic differences at the mouse β -globin locus.¹⁹ Recipients for gene transfer experiments were genetically anemic WBB6F₁-W/W^m mice. Recipients for the CFU-S assay²⁰ were lethally irradiated (900 rad) WBB6F₁-+/+ mice. Recipients for the competitive repopulation assay²¹ were either WBB6F₁-W/W^m mice or lethally irradiated (950 rad) WBB6F₁-+/+ mice. No significant differences were observed between irradiated or genetically anemic recipients in the competitive repopulation assay.

Growth factors. Recombinant human (rh) G-CSF, recombinant rat SCF complexed with polyethylene glycol (SCF), recombinant rat SCF (rSCF), and rh interleukin-6 (rhIL-6) were all supplied by Amgen Inc (Thousand Oaks, CA). Endotoxin levels were shown to be less than 2 pg/ μ g of protein. The G-CSF and SCF were supplied at a concentrations of 530 μ g/mL and 1.0 mg/mL, respectively, and stored at 4°C until use. In previous work, Bridell et al¹⁶ showed that a daily dose of 200 μ g/kg/d of hG-CSF and 50 μ g/kg/d of polyethylene glycol rSCF mobilized maximal numbers of hematopoietic progenitor cells into the peripheral blood, including the primitive high proliferative potential colony-forming cell (HPP-CFC). For injection, G-CSF and SCF were combined at a concentration of 40 μ g/mL G-CSF, and 10 μ g/mL SCF in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA). Donor mice (average weight 20 g) were injected daily with 100 μ L subcutaneously. The rSCF and IL-6 were supplied at concentrations of 1.56 mg/mL and 3.0 mg/mL, respectively. The factors were aliquoted and stored at -70°C until use. Recombinant mouse IL-3 was purchased from Pepro Tech (Canton, MA). IL-3 was diluted to a concentration of 1.0 mg/mL in PBS, aliquoted, and stored at -70°C.

Hematology. Peripheral blood was collected from the retro-orbital sinus into heparinized capillary tubes (Thomas Scientific, Swedesboro, NJ). The peripheral blood white blood cell (WBC) count and red blood cell (RBC) count was determined on a Coulter ZM counter (Coulter Electronics, Hialeah, FL). Hematocrits were determined after centrifugation in an IEC (Needham, MA) micro-hematocrit centrifuge. Differential counts were determined by examination of blood smears from five different animals (at least 100 cells counted/animal) stained with Wright's Giemsa.

Harvest of hematopoietic cells. Peripheral blood was collected under sterile conditions into heparinized saline from the retro-orbital sinus using a Pasteur pipette. An average of 1 mL (of an estimated blood volume of 1.8 mL) per mouse was collected. Mononuclear cells were collected after centrifugation through lymphocyte separation medium (LSM; Organon Teknica, Durham, NC) according to the manufacturer's instructions. BM cells were collected by flushing the contents of femurs and tibias into Dulbecco's minimal essential medium with 15% fetal calf serum (FCS) (Hyclone, Logan, UT), 4 mmol L-glutamine, 50 mg/mL penicillin, and 50 mg/mL streptomycin (all from GIBCO, Gaithersburg, MD). Single-cell suspensions were generated by passing the marrow through a 21-gauge needle. Cell counts were performed using a hemacytometer after lysis of the RBCs in 2% acetic acid.

Colony-forming unit-spleen (CFU-S) and competitive repopulation assays. CFU-S assays were performed essentially as described.²⁰ WBB6F₁ mice irradiated with 900 rad from a Cs¹³⁷ source (GammaCell, Atomic Energy of Canada, Toronto, Ontario) were used as recipients. Between 1×10^5 and 1×10^6 peripheral blood mononuclear cells (PBMCs) isolated from cytokine-treated C57BL/6J donors were injected intravenously. Spleens were collected on day 14 postinjection, fixed in Telleyesniczky's fixative (70% ethanol: formaldehyde: acetic acid; 20:1:1), and the foci counted. The competitive repopulation assay was performed as described by Harrison.²¹ PBMCs or BM cells from cytokine-treated C57BL/6 mice were mixed with an identical number of BM cells from untreated HW80. C57BL/6 and HW80 mice have genetically distinguishable hemoglobins at both the protein and DNA levels. C57BL/6 mice are homozygous for the single allele at the mouse β -globin locus. In

C57BL/6 mice the β -globin genes are located on two 10-kb *EcoRI* fragments. HW80 mice are homozygous for the diffuse allele at the mouse β -globin locus. In HW80 mice the β -globin genes are located on 7-kb and 14-kb *EcoRI* fragments.¹⁹ The cell concentrations were adjusted such that each recipient mouse received 1×10^7 cells from each donor. These conditions have been shown to accurately reflect 10-fold differences in repopulating ability and are accurate to the limits of experimental error (3% to 5%).^{22,23} The relative amounts of single and diffuse hemoglobin were determined by electrophoresis on cellulose acetate gels.²⁴ Four months or more posttransplantation, the animals were killed to determine the relative number of the β^{single} and β^{diffuse} alleles in DNA from the BM and thymus. After digestion with *EcoRI* the DNA was analyzed by Southern blot using a 611-bp *BamHI/Pst I* fragment from IVS2 of the mouse β^{major} gene¹⁹ as a probe.

Enrichment of pluripotent hematopoietic stem cells. The enrichment of pluripotent hematopoietic stem cells was performed as previously described.^{13,25} PBMCs from cytokine-treated mice were incubated with a mixture of fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (MoAbs) directed against murine hematopoietic lineage markers (Lin⁻). Cells expressing *c-kit* were identified with the phycoerythrin-conjugated MoAb ACK-4 (a gift of S.I. Nishikawa, Kumamoto, Japan). During fluorescence-activated cell sorter (FACS) enrichment of PHSC, FITC-stained Lin⁺ cells were gated out. Three populations of Lin⁻ cells were observed: Lin⁻, *c-kit*⁺ (>90%); Lin⁻, *c-kit*^{dim} (~9%); and Lin⁻, *c-kit*^{bright} (<0.5%). We have shown previously that all of the pluripotent hematopoietic stem cells in the peripheral blood of cytokine-treated mice are Lin⁻, *c-kit*^{bright}.¹³

Retrovirus-mediated gene transfer. The retrovirus producer cell lines containing the human multidrug resistance gene (*MDR-1*) have been described previously,²⁶ as has the procedure for retrovirus-mediated gene transfer.^{27,28} Briefly, peripheral blood cells from cytokine-treated mice were collected and subjected to one of two gene transfer protocols. In the first protocol, PBMCs from cytokine-treated mice or BM cells from 5-FU-treated mice were suspended at 5×10^5 cells per mL in Dulbecco's modified Eagle's medium (DMEM) with 15% FCS, 4 mmol L-glutamine, 50 mg/mL penicillin, 50 mg/mL streptomycin, 100 ng/mL SCF, 100 ng/mL human IL-6, and 10 ng/mL IL-3. These cells were placed into suspension culture for a 48-hour prestimulation. The cells were then collected, washed, and cultured for an additional 48 hours on a monolayer of retrovirus producer cells in identical medium and growth factors plus 6 μ g/mL polybrene (Sigma, St Louis, MO). In the second protocol, which was only used for PBMCs, the prestimulation step was eliminated and the cells were cultured directly on monolayers of producer cells in the presence of growth factors and polybrene. After transduction the cells were collected and 2×10^6 cells were injected into WBB6F₁-W/W^m recipients.

RESULTS

Effects of G-CSF and SCF treatment on normal and splenectomized mice. Groups of 5 to 10 intact or splenectomized C57BL/6 mice were treated daily for up to 7 days with G-CSF and SCF, or with carrier alone. The recipients were evaluated daily for the peripheral WBC and RBC count, the differential WBC count, and hematocrit. No changes in the RBC or hematocrit were detected in any of the four groups (data not shown). Likewise, no changes in the WBC count or differential count were observed in the animals treated with carrier alone (Fig 1). In contrast, there was a significant ($P < .01$) increase in the WBCs of splenectomized animals treated for 2 or more days and of intact animals treated for 4 or more days with G-CSF and SCF. The

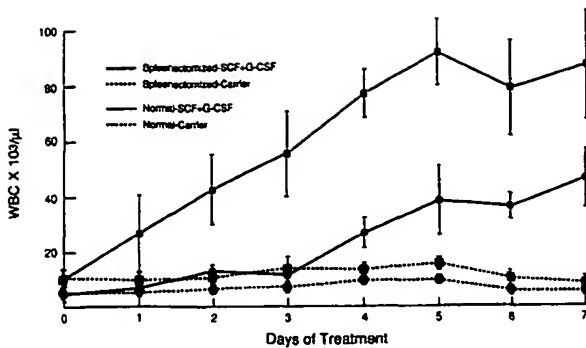


Fig 1. Treatment of mice with G-CSF and SCF increases the WBC count. Splenectomized (■) or Intact (●) mice were treated with daily doses of 200 μ g/kg G-CSF and 50 μ g/kg SCF in 0.1% BSA (—). Control splenectomized (□) or Intact (○) mice were treated with daily doses of 0.1% BSA (----). Peripheral blood (~50 μ L) was withdrawn daily and analyzed for cell number. The points represent the mean and standard deviation for a minimum of 10 animals observed in at least two separate experiments. There was no change in the RBC count or hematocrit in any group of animals.

response to G-CSF and SCF treatment was particularly pronounced in the splenectomized animals, reaching a maximum of 94.7×10^3 cells/ μ L after 5 days of treatment, whereas a maximum of 37.2×10^3 cells/ μ L was achieved after 7 days of treatment in the intact mice (Fig 1). These results differ from those of Bridell et al¹⁶ in that they use a different strain of mice (C57BL/6 as opposed to B6D2F1) and the time course of the increase in WBCs is depicted.

Most of the increase in the WBCs was caused by an increase in the number of peripheral blood granulocytes, the percentage of which increased almost fourfold during the course of treatment (Table 1; $P < .001$). The frequency of CFU-S in the peripheral blood of untreated splenectomized mice was higher than in the peripheral blood of intact mice ($0.1 \text{ v } 1.9$; $P < .01$). The frequency of CFU-S in the peripheral blood of splenectomized mice treated with G-CSF and SCF increased significantly after 3 days of treatment, reach-

ing a maximum of 19.0 ± 4.9 CFU-S/ 10^5 cells injected after 7 days (Table 1; $P < .001$).

Effects of G-CSF and SCF treatment on the repopulating ability of peripheral blood and BM cells. To assay for pluripotent hematopoietic stem cells, a competitive repopulation assay was used. C57BL/6J mice, either intact or splenectomized, were treated for various lengths of time with G-CSF and SCF. Peripheral blood and BM cells were collected from these animals and mixed with an equal number of BM cells from intact untreated HW80 mice. C57BL/6J and HW80 mice are genetically identical except at the β -globin locus, where C57BL/6J mice are homozygous for the single allele, and HW80 mice homozygous for the diffuse allele. The 1:1 cell mixtures are then injected into histocompatible recipients, either lethally irradiated WBB6F₁-+/+ or genetically anemic WBB6F₁-W/W^v (W/W^v) mice, such that each recipient received 1×10^7 cells from each donor (Fig 2). After reconstitution with the transplanted cells the relative number of RBCs derived from each of the donor strains can be measured by quantifying the amount of single and diffuse hemoglobin in the peripheral blood. The relative repopulating ability is expressed as the ratio of the amount of C57BL/6J-derived hemoglobin to the total amount of C57BL/6J- and HW80-derived hemoglobin. Likewise, the relative number of lymphoid and myeloid cells derived from each donor can be established by assaying for the relative amount of the single and diffuse hemoglobin alleles in DNA extracted from the thymus and BM using the *Eco*RI restriction fragment length polymorphisms.²⁹ Previous studies have shown that when greater than 10^6 cells are injected this assay is quantitative and accurate to the limits of an experimental error of 3% to 5%.^{22,23}

BM cells from untreated splenectomized mice and untreated intact mice repopulate equivalently, resulting in approximately 50% of single and diffuse hemoglobin in the repopulated recipients (Day 0, Fig 3). Treatment of splenectomized mice with G-CSF and SCF resulted in an initial increase in repopulating ability relative to untreated marrow after 3 days of treatment ($P < .01$) followed by a decrease

Table 1. Effects of G-CSF and SCF Treatment on the Number and Distribution of Peripheral Blood Cells

Day	WBC*	% Lymphocytes	% Granulocytes	% Monocytes	CFU-S ₁₄ 10 ⁶ PBMCs
Splenectomized mice					
0	10.7 \pm 2.9	80.6 \pm 0.9	15.6 \pm 1.1	3.8 \pm 0.8	1.9 \pm 1.0
1	12.8 \pm 3.5	65.6 \pm 5.9†	31.6 \pm 7.1†	3.0 \pm 1.6	ND
3	57.4 \pm 13.5†	55.8 \pm 3.1†	39.8 \pm 7.4†	4.8 \pm 0.9	7.8 \pm 1.0†
5	94.7 \pm 8.9†	42.6 \pm 2.3†	54.8 \pm 3.1†	2.4 \pm 1.1	13.1 \pm 3.3†
7	89.9 \pm 16.8†	39.8 \pm 5.9†	58.4 \pm 4.2†	1.8 \pm 2.5	19.0 \pm 4.9†
Normal mice					
0	5.3 \pm 1.8	78.2 \pm 1.5	18.4 \pm 1.3	3.4 \pm 0.7	0.1 \pm 0.2
1	6.9 \pm 1.8	72.7 \pm 3.1	24.0 \pm 4.1†	3.3 \pm 1.1	ND
3	12.0 \pm 2.6†	67.4 \pm 4.3†	29.2 \pm 5.1†	3.4 \pm 0.8	ND
5	39.4 \pm 11.4†	62.0 \pm 5.5†	34.1 \pm 7.9†	3.9 \pm 1.4	ND
7	47.3 \pm 9.5†	56.8 \pm 6.2†	40.9 \pm 5.6†	2.3 \pm 1.7	ND

n \geq 10 for each WBC, representing at least two separate experiments. n $>$ 5 for % lymphocytes, granulocytes, and monocytes, representing at least two separate experiments. n $>$ 25 CFU-S colonies located on at least 10 spleens in two separate experiments.

Abbreviation: ND, not done.

* Number of WBCs $\times 10^6$ /mL.

† $P < .001$ relative to day 0.

Competitive Repopulation Assay

Fig 2. The competitive repopulation assay. In this example the donor mouse is C57BL/6J, which is homozygous for "single" hemoglobin (shown at the upper left of the figure), and is treated with G-CSF and SCF daily. PBMCs or BM cells are collected from mice after 0, 1, 3, 5, or 7 days of treatment and mixed with an equal number of BM cells from untreated HW80 mice. The HW80 strain is congenic with C57BL/6J and is homozygous for "diffuse" hemoglobin (shown at the middle left of the figure). The cell mixtures are injected into either irradiated WBB6F1-+/+ or genetically anemic WBB6F1-W/W^u recipients that have a "single/diffuse" hemoglobin pattern (heterozygous for "single" and "diffuse" hemoglobin, shown at the lower left of the figure). The proportion of "single" and "diffuse" hemoglobin present after complete repopulation (4 months) is proportional to the relative number of pluripotent hematopoietic stem cells injected.^{21,22}

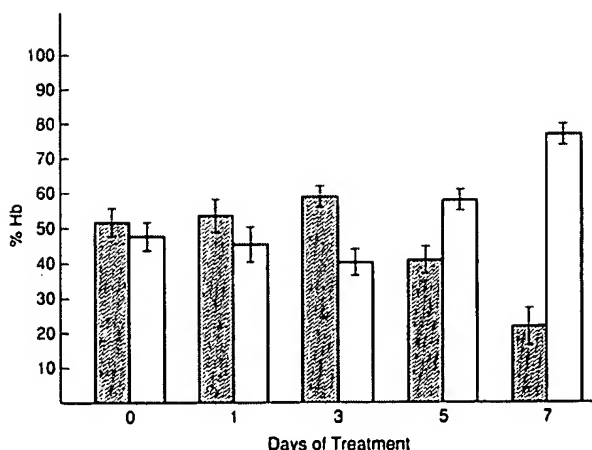
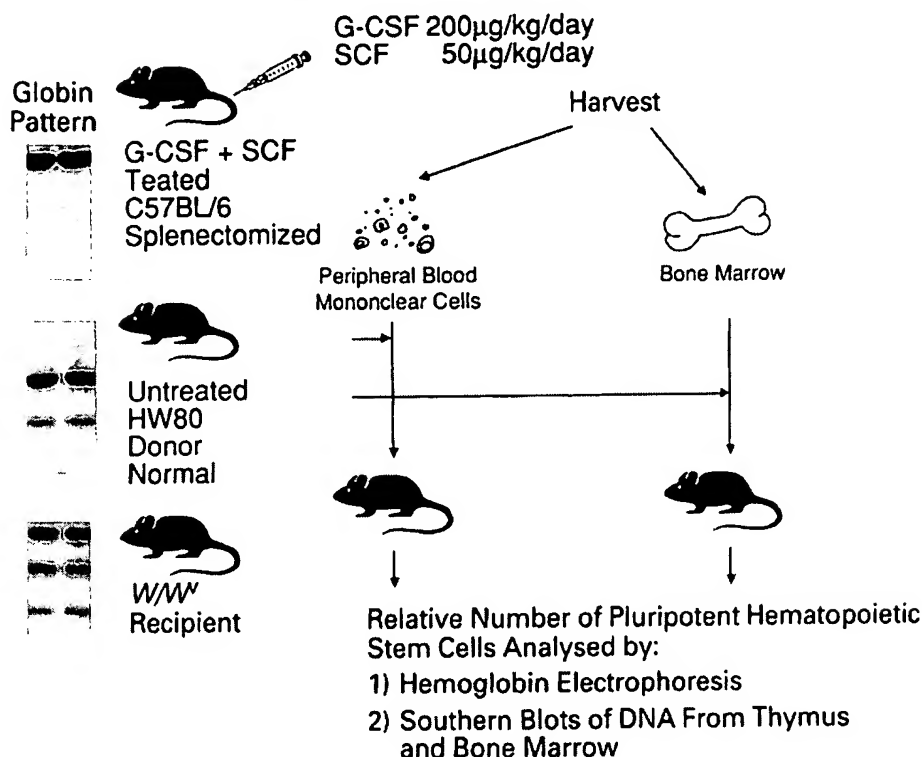


Fig 3. Competitive repopulating ability of BM cells from splenectomized mice treated with G-CSF and SCF (■) versus BM cells from intact untreated mice (□). BM cells were collected from splenectomized C57BL/6J mice (single hemoglobin) after the indicated days of treatment and competed against BM cells from HW80 mice (diffuse hemoglobin). The percentage and standard deviation of single and diffuse hemoglobins present in the peripheral blood 4 months post-transplantation are shown. Each bar represents the average of at least 10 mice from at least two separate experiments.

in the repopulating ability after 5 and 7 days of treatment (Fig 3 and Table 2; $P < .001$). The repopulating ability of BM from splenectomized mice decreased to less than 30% of the normal level after 7 days of treatment. Similar, but less pronounced, effects were observed in intact mice.

The amount of hemoglobin derived from peripheral blood cells from untreated intact or splenectomized mice was less than 20% of that of BM cells from untreated intact mice. After 3 days of treatment with G-CSF and SCF, the amount of hemoglobin derived from peripheral blood cells from untreated intact or splenectomized mice increased significantly ($P < .01$), indicating an increase in the number of PHSC in the peripheral blood in response to treatment with G-CSF and SCF (Fig 4 and Table 2). In splenectomized mice the amount of hemoglobin derived from peripheral blood cells from mice treated for 3 to 7 days exceeded that of a similar number of BM cells (Fig 4 and Table 2; $P < .01$). The greatest concentration of peripheral blood PHSC was observed in both intact and splenectomized mice after 5 days of treatment (Fig 4 and Table 2; $P < .001$). This effect was most pronounced in the splenectomized mice where over 90% of the hemoglobin was derived from peripheral blood PHSC (Table 2). Southern blot analysis of DNA from the BM and thymus showed that the relative ratios of the β^{single} and β^{major} alleles was similar to the relative ratio of single and diffuse hemoglobin²⁹ (and data not shown).

The ratio of single to diffuse hemoglobin can be used to calculate the repopulating ability of a given population of cells relative to normal BM cells.²³ Multiplying the relative

Table 2. Effects of G-CSF and SCF Treatment on the Repopulating Ability of Peripheral Blood and Bone Marrow Cells

Day	Normal		Splenectomized	
	% Hb From Cytokine-Treated Peripheral Blood	% Hb From Untreated Marrow	% Hb From Cytokine-Treated Peripheral Blood	% Hb From Untreated Marrow
0	12.4 ± 3.6	87.6 ± 3.6	15.3 ± 3.2	84.7 ± 3.2
1	ND	ND	21.7 ± 8.1	78.3 ± 8.1
3	20.5 ± 6.9*	79.5 ± 6.9	80.6 ± 3.7†	19.4 ± 3.7
5	36.9 ± 5.7†	63.1 ± 5.7	90.1 ± 4.5†	9.9 ± 4.5
7	32.4 ± 4.9†	67.6 ± 4.9	80.1 ± 5.7†	19.9 ± 5.7
Day	% Hb From Cytokine-Treated Marrow	% Hb From Untreated Marrow	% Hb From Cytokine-Treated Marrow	% Hb From Untreated Marrow
0	51.1 ± 4.0	48.9 ± 4.0	52.3 ± 4.3	47.7 ± 4.3
1	ND	ND	54.3 ± 4.1	45.7 ± 4.1
3	56.8 ± 4.9	43.2 ± 4.9	59.4 ± 3.4*	40.6 ± 3.4
5	46.2 ± 4.7	53.8 ± 4.7	41.7 ± 3.0†	58.3 ± 3.0
7	26.9 ± 4.1†	73.1 ± 4.1	22.5 ± 4.9†	77.5 ± 4.9

The percentage of hemoglobin (Hb) derived from hematopoietic cells from cytokine-treated mice (C57BL/6 single Hb) and competitor BM from untreated mice (HW80 diffuse Hb) was determined after cellulose acetate electrophoresis. Each data point represents the average of at least 10 mice from at least two separate experiments.

Abbreviation: ND, not done.

* $P < .01$ relative to day 0.

† $P < .001$ relative to day 0.

repopulating ability by the estimated frequency of PHSC in normal BM generates an estimated frequency of PHSC in the test population (see example in the legend of Table 3). The frequency of PHSC in the peripheral blood and BM cells from splenectomized mice treated for 5 days with G-CSF and SCF was 9.01 and 0.71 PHSC/ 10^5 cells, respectively (Table 3).

Effects of G-CSF and SCF treatment on the absolute number of hematopoietic stem cells/mouse. The PHSC frequen-

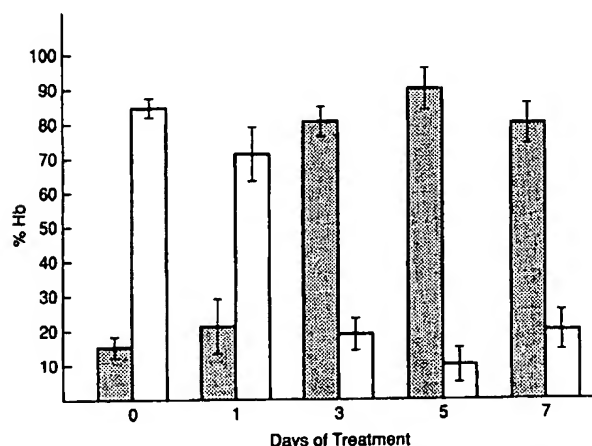


Fig 4. Competitive repopulating ability of PBMCs from splenectomized mice treated with G-CSF and SCF (■) versus BM cells from intact untreated mice (□). PBMCs were collected from splenectomized C57BL/6J mice (single hemoglobin) after the indicated days of treatment and competed against BM cells from HW80 mice (diffuse hemoglobin). The percentage and standard deviation of single and diffuse hemoglobins present in the peripheral blood 4 months post-transplantation are shown. Each bar represents the average of at least 10 mice from at least two separate experiments.

cies were multiplied by the total number of PBMCs and BM cells per mouse to estimate the absolute number of PHSC per mouse. The number of peripheral blood cells was calculated from the WBC and differential counts in Table 1, based on a blood volume of 1.8 mL/mouse. The BM cellularity of mice treated with G-CSF and SCF was unchanged relative to normal intact mice, so the estimate of 2.4×10^8 BM cells/mouse calculated by Chervenick et al³⁰ was used. Using these calculations we previously showed that normal mice have an estimated 3,000 stem cells, with approximately 2,400 in the marrow, 550 in the spleen, and less than 10 in the peripheral blood.¹³ Untreated splenectomized mice have slightly fewer PHSC because of the removal of the spleen (Table 3). With treatment of splenectomized mice for 5 days with G-CSF and SCF, the absolute number of PHSC increased to approximately 9,000, with over 7,000 in the peripheral blood (Table 3).

We have previously shown that PHSC and CFU-S from BM and peripheral blood can be highly enriched by selecting for cells that do not express markers for the erythroid, myeloid, and lymphoid lineages (Lin^-), and express high levels of *c-kit* (*c-kit*^{BR}).^{13,25} The percentage and absolute number of cells with the Lin^- , *c-kit*^{BR} phenotype in the peripheral blood of splenectomized mice treated with G-CSF and SCF increased and decreased in parallel with the number of PHSC and CFU-S (Table 3). These results establish that treatment with G-CSF and SCF increases both the number of peripheral blood PHSC (as measured by the competitive repopulation assay) and the number of circulating cells with the Lin^- , *c-kit*^{BR} phenotype (among which are concentrated CFU-S and PHSC).

Gene transfer into peripheral blood PHSC mobilized by G-CSF and SCF. The efficiency of gene transfer into PHSC mobilized into the peripheral blood by treatment with G-CSF and SCF was compared with gene transfer into PHSC

Table 3. Estimation of the Total Number of Pluripotent Hematopoietic Stem Cells in Splenectomized Mice Treated With G-CSF and SCF

Day	Tissue	Relative Repopulating Activity*	Cellularity†	Estimated No. PHSC‡	% Lin ⁺ , c-kit ^{hi} Cells	
					Exp 1	Exp 2
0	PBMC	0.18	1.8×10^7	29	0.01	0.01
	BM	1.09	2.4×10^8	2,616		
				Total 2,645		
1	PBMC	0.28	1.6×10^7	45	ND	ND
	BM	1.19	2.4×10^8	2,856		
				Total 2,901		
3	PBMC	4.15	6.8×10^7	2,822	0.6	0.3
	BM	1.46	2.4×10^8	3,504		
				Total 6,326		
5	PBMC	9.01	8.0×10^7	7,208	2.2	1.1
	BM	0.71	2.4×10^8	1,704		
				Total 8,912		
7	PBMC	4.03	6.8×10^7	2,740	0.6	0.5
	BM	0.29	2.4×10^8	696		
				Total 3,436		
Untreated	PBMC	0.14	7.0×10^6	10		
Normal	SP	0.24	2.3×10^8	550		
	BM	1.0	2.4×10^8	2,400		
				Total 2,960		

Abbreviations: SP, spleen cells; ND, not done.

* Calculated from the data in Figs 3 and 4 and Table 2.

$$\frac{\% \text{Hb From Experimental Population}}{\% \text{Hb From Control Population}} = \text{Relative Repopulating Ability}$$

This value multiplied by the estimated frequency of PHSC in the BM of normal mice (1 PHSC/ 10^6 cells) gives an estimated frequency of PHSC in the experimental population. Example:

$$\frac{\% \text{Hb Day 7 PBMC (80.1)}}{\% \text{Hb Normal BM (19.9)}} \times \frac{1 \text{ PHSC}}{10^6 \text{ BM Cells}} = 4.03 \text{ PHSC}/10^6 \text{ Day 7 PBMC}$$

† Total number of nucleated cells per mouse in the indicated tissues. The peripheral blood values were calculated from the data in Table 1, using an estimated blood volume of 1.8 mL/mouse. For BM the estimate of Chervenick et al³⁰ was used. No differences in the cellularity of normal, splenectomized, or cytokine-treated mice were observed. Normal values were presented in reference 13.

‡ Calculated by multiplying the estimated frequency of PHSC (see* note) by the cellularity. Example:

$$\frac{4.03 \text{ PHSC}}{10^6 \text{ Day 7 PBMC}} \times \frac{6.8 \times 10^7}{\text{Day 7 PBMC}} = 2,740 \text{ PHSC in Day 7 Peripheral Blood}$$

present in 5-FU-treated BM cells, which we and others have shown is superior for gene transfer.^{27,31,32} 5-FU BM cells and cells collected after treatment with G-CSF and SCF were cocultured with one of two retrovirus producer cell lines, each containing the human multidrug resistance gene (*MDR-1*).²⁶ In the 18.1 cell line, the *MDR-1* gene is contained in a Harvey sarcoma virus backbone, whereas in the Eco-8 cell line, the *MDR-1* gene is contained in a Moloney murine leukemia virus backbone. We also compared two protocols for transducing peripheral blood PHSC. The first protocol is identical to the protocol described for 5-FU-treated marrow, and it includes a 48-hour prestimulation in suspension culture in the presence of growth factors followed by a 48-hour coculture with the producer cells. The second protocol omits the prestimulation step, and the peripheral blood cells are cocultured directly with the producer cells for 48 hours. We have shown previously that omitting the prestimulation reduces gene transfer to BM PHSC by over 10-fold.²⁸ Our rationale for omitting prestimulation for peripheral blood PHSC transduction was to test the hypothesis that the cyto-

kine treatment in vivo had prepared the cells for optimal transduction.

After transduction, the cells were returned to *W/W^v* recipient mice, which were analyzed for the presence and copy number of the *MDR* provirus 4 months posttransplantation by polymerase chain reaction (PCR) analysis of peripheral blood DNA using primers specific for the human *MDR-1* gene. Work by several groups including ourselves has shown that results observed at 4 months are caused by PHSC and not by short-term progenitor cells.^{22,23,27,28,33}

Gene transfer was observed in 32 of 33 recipients of transduced 5-FU BM cells (Table 4), which showed that our producer cells performed reliably from experiment to experiment. Regardless of the producer cell line or the protocol used, negligible gene transfer (2/60) was observed in the recipients of transduced peripheral blood cells from untreated mice or from mice treated for 1 day with G-CSF and SCF (Table 4). Efficient gene transfer was observed in the recipients of transduced peripheral blood cells collected after 3 to 7 days of treatment with G-CSF and SCF (Table 4).

Table 4. Gene Transfer Into Peripheral Blood PHSC Mobilized by Treatment of Spenectomized Mice With G-CSF and SCF

Exp.	Virus	5-FU Marrow	Protocol	No. Mice Containing <i>MDR</i> Proviral Sequences*				
				No. Mice Injected				
				Days of Treatment				
				0	1	3	5	7
1	MDR 18.1	5/5	Prestim + coculture			10/10	10/10	11/12
2	MDR 18.1	6/6	Prestim + coculture	0/5		4/4	8/8	12/12
3	MDR Eco 8	5/5	Prestim + coculture	0/12	1/10	8/9	17/17	
4	MDR 18.1	5/5	Direct coculture			5/12	11/12	8/8
5	MDR Eco 8	5/6	Direct coculture	0/6	0/6	3/8	8/8	
6	MDR Eco 8	6/6	Direct coculture	1/6		4/7	6/6	

* Data collected 4 months posttransplantation.

The omission of the prestimulation step caused the gene transfer efficiency to drop to 50% positive recipients when cells were collected after 3 days of treatment with G-CSF and SCF, but had little or no effect on gene transfer into cells collected after 5 or 7 days of treatment (Table 4).

The screen for proviral sequences described above does not assay for copy number and may reflect efficient gene transfer to many PHSC or inefficient gene transfer to a small number of PHSC. Inspection of the PCR signals suggested that the intensity of the *MDR-1* signal was greater in the DNA from mice repopulated with cells collected after 5 or 7 days of treatment with G-CSF and SCF (Fig 5). This observation was confirmed by a reanalysis of the animals in experiments 1 and 2 in Table 4 including the concurrent 5-FU BM controls and an additional 10 5-FU BM controls from another experiment. Peripheral blood DNA from these animals was coamplified using primers specific for a portion of the endogenous mouse β -globin gene and the human *MDR-1* gene. Using a phosphorimager the relative intensities of the β -globin and *MDR* signals could be quantified for an estimation of the number of proviral copies per cell. Mice repopulated with transduced 5-FU BM cells contained an average of 0.23 ± 0.12 copies of the *MDR-1* provirus per cell (Table 5), which is similar to the results we have published previously.²⁶ Mice repopulated with transduced peripheral blood cells collected after 5 and 7 days of treatment had contained significantly higher average proviral copy numbers of 0.54 ± 0.23 and 0.75 ± 0.75 , respectively ($P < .01$ in both cases; Table 5).

Southern blot analysis was performed on DNA extracted from the animals in experiments 1 and 2 in Table 4. Recipients transplanted with peripheral blood cells mobilized by G-CSF and SCF treatment for 5 to 7 days showed multiple proviral insertion sites that were shared in DNA extracted from both the BM and thymus. The number of insertions was twofold to fivefold higher than that observed in mice repopulated with 5-FU-treated BM cells (data not shown). The same DNAs were probed with a mouse β -globin probe to assess the extent of repopulation in the lymphoid and myeloid compartments. The recipient *W/W^v* mice are heterozygous at the β -globin locus and have both the 10-kb band of the single haplotype and the 7-kb band of the diffuse haplotype, whereas the homozygous C57BL/6 donor mice have only the 10-kb band of the single haplotype (Fig 6).

The repopulated recipients have only the 10-kb band of the C57BL/6 donor in both the BM and thymus, showing complete lymphoid and myeloid repopulation by the cells exposed to retrovirus (Fig 6). The erythroid compartment was also 100% donor derived as evidenced by the presence of only single hemoglobin in the repopulated recipients.

DISCUSSION

The ability of cytokines and/or cytotoxic drugs to mobilize hematopoietic progenitor cells into the peripheral blood of experimental animals and humans is well documented.⁷ Mobilized peripheral blood PHSC could be an alternative to BM PHSC for gene-therapy protocols, allowing multiple collections of PHSC or as a means of collecting larger numbers of PHSC from patients with fibrotic BM.¹⁵ Several requirements must be met before cytokine-mobilized peripheral blood hematopoietic progenitor cells can be considered for gene-therapy protocols. First, mobilized peripheral blood cells must be shown to contain pluripotent hematopoietic stem cells capable of repopulating the lymphoid, myeloid, and erythroid compartments of an ablated recipient. Secondly, the mobilization should not compromise the donor by reducing the number of PHSC per individual. Finally, gene transfer into mobilized peripheral blood PHSC must be at least as efficient, if not more efficient, than gene transfer into BM PHSC.³⁴

The results we have described here address all three of these points. The effects on peripheral blood cell counts in mice treated with the combination of G-CSF and SCF are identical to those published previously by Molineux et al¹⁸ and Bridell et al.¹⁶ Our results extend the previous findings by providing a time course for the increase in WBCs, which can be used to predict the optimal time to collect peripheral blood PHSC in large animals and patients. We have also used a quantitative repopulation assay to measure the number of repopulating stem cells in the peripheral blood and marrow rather than the radioprotection assays used in earlier work. In this study, as in our previous work, we have focused on the hematopoietic repopulating cell (PHSC) that is analyzed 4 months posttransplantation.^{22,23,27,28,33} Our results show that peripheral blood cells from animals treated with SCF alone and with G-CSF and SCF contain cells capable of repopulating the erythroid, myeloid, and lymphoid compartments of *W/W^v* mice. We believe that these results cou-

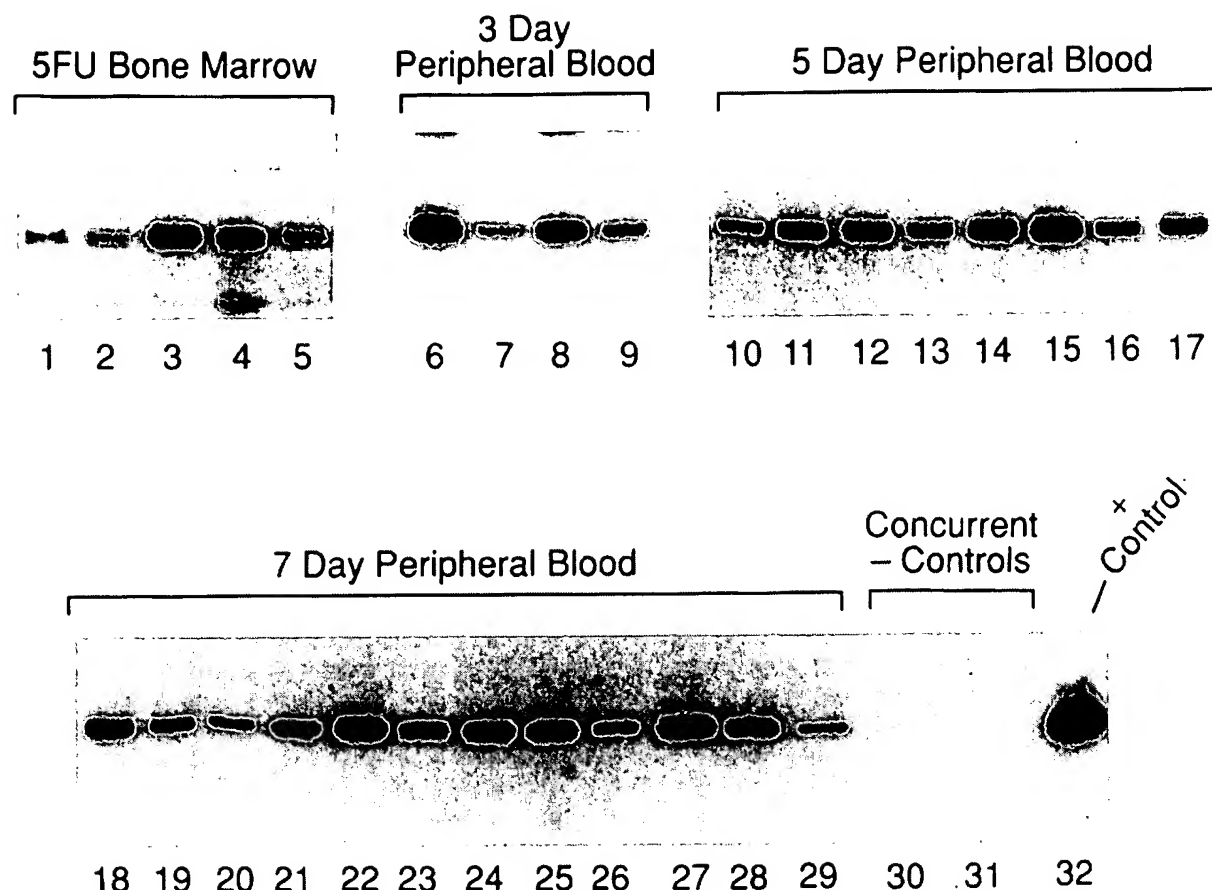


Fig 5. Gene transfer into mobilized peripheral blood PHSC from splenectomized mice. The figure shows a PCR analysis of DNA extracted from the peripheral blood of mice repopulated with cells transduced with a virus containing the human MDR gene. The DNA samples analyzed in this figure represent experiment 2 in Table 4. Lanes 1 through 5, DNA extracted from mice repopulated with transduced BM from 5-FU-treated donor mice; lanes 6 through 9, DNA extracted from mice repopulated with transduced peripheral blood cells collected after 3 days of treatment with G-CSF and SCF; lanes 10 through 20, DNA extracted from mice repopulated with transduced peripheral blood cells collected after 5 days of treatment with G-CSF and SCF; lanes 21 through 30, DNA extracted from mice repopulated with transduced peripheral blood cells collected after 7 days of treatment with G-CSF and SCF; lanes 31 and 32, DNA extracted concurrently from negative control animals. Amplification of the same DNA samples with primers specific for the mouse β -globin gene indicated that all lanes were loaded equivalently (see Table 4).

pled with the presence of shared proviral insertion sites in multiple hematopoietic organs establishes gene transfer to pluripotent hematopoietic stem cells.

Although the absolute number of PHSC in the BM decreases after 5 to 7 days of treatment, the absolute number of PHSC in the animal increases threefold. These results establish that the PHSC mobilized into the peripheral blood by treatment with the combination of G-CSF and SCF are the result of an expansion of the overall number of PHSC in the animal, which more than compensate for the reduction in the number of BM PHSC.

The effects of G-CSF and SCF treatment on the WBC count and the number of peripheral blood hematopoietic colony-forming cells are greater in splenectomized mice treated with G-CSF and SCF than in intact mice.^{10,11,13,16,18} In this report we show that the overall number of peripheral blood PHSC per splenectomized mouse increases nearly threefold in response to treatment with G-CSF and SCF over untreated splenectomized mice. In previous studies we

showed that in intact mice treated with SCF alone, most of the hematopoietic expansion was in the spleen, which is a major site of extramedullary hematopoiesis in the mouse.¹³ We propose that the removal of the spleen allows a greater

Table 5. Estimated Copy Number of MDR Proviral Sequences in Mice Repopulated With Transduced Peripheral Blood PHSC From Splenectomized Mice Treated With G-CSF and SCF

Cell Source	Average No. Copies/Cell*	n	P
5-FU Marrow	0.23 ± 0.19	21	
3 day PB	0.08 ± 0.06	8	.051
5 day PB	0.54 ± 0.15	16	.02
7 day PB	0.75 ± 0.75	21	.003

Data in this table are derived from the animals in experiments 1 and 3 in Table 4.

Abbreviation: PB, peripheral blood.

*Data collected 4 months posttransplantation.

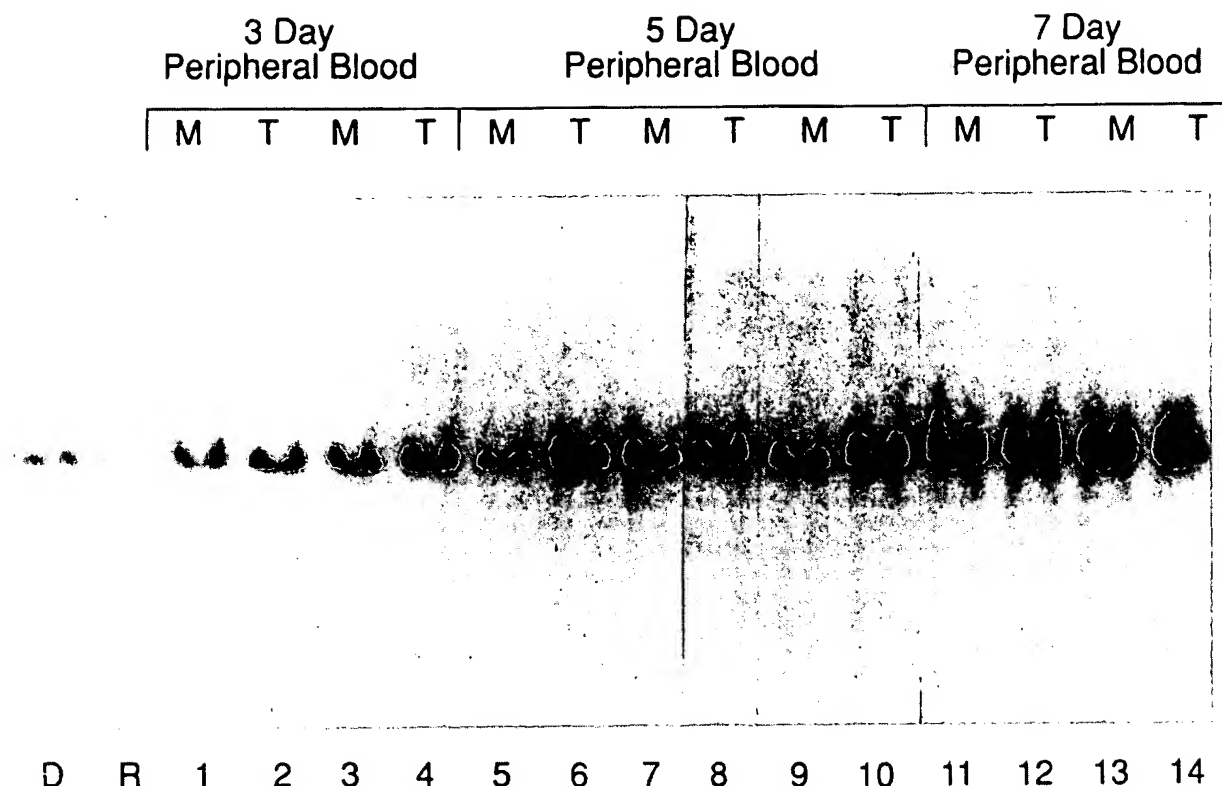


Fig 6. Southern blot analysis of DNA extracted from repopulated mice. DNA was extracted from the BM and thymus of mice repopulated with peripheral blood cells collected after the indicated days of treatment with G-CSF and SCF, digested with *EcoRI*, and probed with a probe for the IVS2 of the mouse β -globin gene. The C57BL/6J donor (D) has comigrating 10-kb bands, whereas the recipient (R) is heterozygous and has the 10-kb "single" bands, and the 14.0-kb and 7.0-kb "diffuse" bands. Odd-numbered lanes, BM DNA; even-numbered lanes, thymus DNA. Lanes 1 through 6, DNA from mice repopulated with peripheral blood cells collected after 3 days of treatment with G-CSF and SCF; lanes 7 through 14, DNA from mice repopulated with peripheral blood cells collected after 5 days of treatment with G-CSF and SCF; lanes 15 through 20, DNA from mice repopulated with peripheral blood cells collected after 7 days of treatment with G-CSF and SCF.

redistribution of PHSC into the peripheral blood. In larger animals the spleen is not a major site of extramedullary hematopoiesis. It is unclear whether splenectomy will be required to obtain the maximum number of peripheral blood PHSC in larger animals or human patients.

It has been shown that gene transfer efficiency into human CD34⁺ progenitor cells collected from patients treated with cytokines and chemotherapy is greater than into CD34⁺ progenitor cells from BM.^{14,15} Previously we showed that mouse peripheral blood and spleen PHSC mobilized by treatment with SCF alone could be marked with retroviruses, but that the frequency of gene transfer was lower than the frequency of gene transfer into BM cells from 5-FU-treated mice.¹³ Treatment of splenectomized mice with G-CSF and SCF mobilizes large numbers of PHSC into the peripheral blood that are more efficiently marked by retroviruses than PHSC from the bone marrow of 5-FU-treated mice. We conclude that gene transfer into peripheral blood cells from patients treated with G-CSF and SCF may be as efficient or more efficient than gene transfer into human BM PHSC. Given the low rate of gene transfer into BM observed in primate and canine model systems,³⁴⁻³⁸ cytokine-mobilized peripheral

blood stem cells present an exciting alternative target for retroviral transduction.

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REFERENCES

1. Micklem HS, Anderson N, Ross E: Limited potential of circulating stem cells. *Nature* 256:41, 1975
2. Duhrsen U, Villeval JL, Boyd J, Kannourakis G, Morstyn G, Metcalf D: Effects of recombinant human granulocyte colony stimulating factor on hematopoietic progenitor cells in cancer patients. *Blood* 72:2074, 1988
3. Sicna S, Bregni M, Brando B, Ravagnani F, Bonadonna G, Gianni AM: Circulation of CD34⁺ hematopoietic stem cells in the peripheral blood of high-dose cyclophosphamide-treated patients: Enhancement by intravenous recombinant granulocyte-macrophage colony-stimulating factor. *Blood* 74:1905, 1989
4. Lasky LC: Hematopoietic reconstitution using progenitors recovered from blood. *Transfusion* 29:552, 1989

5. Gianni AM, Tarella C, Siena S, Bregni M, Boccadoro M, Lombardi F, Bengala C, Bonadonna G, Pileri A: Durable and complete hematopoietic reconstitution after autografting of rhGM-CSF exposed peripheral blood progenitor cells. *Bone Marrow Transplant* 6:143, 1990
6. Pileri A, Tarella C, Bregni M, Boccadoro M, Siena S, Caracciolo D, Bonadonna G, Gianni AM: GM-CSF exposed peripheral blood progenitors as sole source of stem cells for autologous transplantation in two patients with multiple myeloma. *Hematologia* 75:79, 1990
7. Eaves CJ: Peripheral blood stem cells reach new heights. *Blood* 82:1957, 1993
8. Weaver CH, Buckner CD, Longin K, Appelbaum FR, Rowley S, Lilleby K, Miser J, Storb R, Hansen JA, Besinger W: Syngeneic transplantation with peripheral blood mononuclear cells collected after the administration of recombinant human granulocyte colony-stimulating factor. *Blood* 82:1981, 1993
9. Molineux G, Pojda Z, Dexter TM: A comparison of haemopoiesis in normal and splenectomized mice treated with G-CSF. *Blood* 75:563, 1990
10. Pettengell R, Testa NG, Swindell R, Crowther D, Dexter TM: Transplantation potential of hematopoietic cells released into the circulation during routine chemotherapy for non-Hodgkin's lymphoma. *Blood* 82:2239, 1993
11. Bungart B, Loeffler M, Goris H, Dontje B, Diehl V, Nijhof W: Differential effects of recombinant human colony stimulating factor (rhG-CSF) on stem cells in marrow spleen and peripheral blood in mice. *Br J Haematol* 76:174, 1990
12. Andrews RG, Bartelmez SH, Knitter GH, Myerson D, Bernstein ID, Applebaum FR, Zsebo KM: A c-kit ligand, recombinant human stem cell factor, mediates reversible expansion of multiple CD34⁺ colony forming cell types in blood and marrow of baboons. *Blood* 80:920, 1992
13. Bodine DM, Seidel NE, Zsebo KM, Orlie D: In vivo administration of stem cell factor to mice increases the absolute number of pluripotent hematopoietic stem cells. *Blood* 82:445, 1993
14. Cassel A, Cottler-Fox M, Doren S, Dunbar CE: Retroviral-mediated gene transfer into CD34-enriched human peripheral blood stem cells. *Exp Hematol* 21:585, 1993
15. Bregni M, Magni M, Siena S, DiNicola M, Bonadonna G, Gianni AM: Human Peripheral blood hematopoietic progenitors are optimal targets of retroviral-mediated gene transfer. *Blood* 80:1418, 1992
16. Bridell RA, Hartley CA, Smith KA, McNiece IK: Recombinant rat stem cell factor synergizes with recombinant human granulocyte-colony stimulating factor in vivo in mice to mobilize peripheral blood progenitor cells that have enhanced repopulating ability. *Blood* 82:1720, 1993
17. Molineux G, Pojda Z, Hampson IN, Lord BI, Dexter TM: Transplantation potential of peripheral blood stem cells induced by granulocyte colony-stimulating factor. *Blood* 76:2153, 1990
18. Molineux G, Migdalska A, Szmitkowski M, Zsebo K, Dexter TM: The effects of hematopoiesis of recombinant stem cell factor (ligand for c-kit) administered in vivo to mice either alone or in combination with granulocyte colony-stimulating factor. *Blood* 78:961, 1991
19. Weaver S, Haigwood NL, Hutchison CA, Edgell MH: DNA fragments of the *Mus musculus* β globin haplotypes. *Proc Natl Acad Sci USA* 76:1385, 1979
20. Till JE, McCulloch EA: A direct measure of the radiation sensitivity of normal mouse cells. *Radiat Res* 14:213, 1961
21. Harrison DE: Competitive repopulation: A new assay for long term stem cell capacity. *Blood* 55:77, 1980
22. Harrison DE, Jordan CT, Zhong RK, Astle CM: Primitive hematopoiesis stem cells: Direct assay of most productive populations by competitive repopulation with simple binomial, correlation and covariance calculations. *Exp Hematol* 21:206, 1993
23. Harrison DE, Astle CM, Lerner C: Number and continuous proliferative pattern of transplanted primitive immunohematopoietic stem cells. *Proc Natl Acad Sci USA* 85:822, 1988
24. Whitney JB III: Simplified typing of mouse hemoglobin (Hbb) phenotypes using cystamine. *Biochem Genet* 16:667, 1978
25. Orlie D, Fischer R, Nishikawa S-I, Nienhuis AW, Bodine DM: Purification and characterization of heterogeneous pluripotent hematopoietic stem cell populations expressing high levels of c-kit receptor. *Blood* 82:762, 1993
26. Sorrentino BP, Brandt SJ, Bodine D, Gottesman M, Pastan I, Cline A, Nienhuis AW: Selection of drug resistant bone marrow cells *in vivo* after retroviral transfer of human MDR1. *Science* 257:99, 1992
27. Bodine DM, McDonagh KT, Seidel NE, Nienhuis AW: Survival and retrovirus infection of murine hematopoietic stem cells *in vitro*: Effects of 5-FU and method of infection. *Exp Hematol* 19:206, 1990
28. Bodine DM, Karlsson S, Nienhuis AW: Combination of interleukins 3 and 6 preserves stem cell function in culture and enhances retrovirus mediated gene transfer into hematopoietic stem cells. *Proc Natl Acad Sci USA* 86:8897, 1989
29. Bodine DM, Orlie D, Birkett NC, Seidel NE, Zsebo KM: Stem Cell factor increases CFU-S number in vitro in synergy with interleukin-6 and in vivo as a single factor. *Blood* 70:913, 1992
30. Chervenick PA, Boggs DR, Marsh JC, Cartwright GE, Wintroub MM: Quantitative studies of blood and bone marrow neutrophils in normal mice. *Am J Physiol* 215:353, 1968
31. Dick JE, Magli MC, Huzar D, Phillips RA, Bernstein A: Introduction of a selectable gene into primitive stem cells capable of long-term reconstitution of the hematopoietic system of W/W^v mice. *Cell* 42:71, 1985
32. Lemischka IR, Raulat DH, Mulligan RC: Developmental potential and dynamic behavior of hematopoietic stem cells. *Cell* 45:917, 1986
33. Jordan CT, Lemischka IR: Clonal and systemic analysis of long term hematopoiesis in the mouse. *Genes Dev* 4:220, 1990
34. Anderson WF: Human gene therapy. *Science* 256:808, 1992
35. Bodine DM, Moritz T, Donahue RE, Luskey BD, Kessler SW, Martin DIK, Orkin SH, Nienhuis AW, Williams DA: Long-term in vivo expression of a murine adenosine deaminase gene in Rhesus monkey hematopoietic cells of multiple lineages after retroviral mediated gene transfer into CD34⁺ bone marrow cells. *Blood* 82:1975, 1993
36. van Beusechem VW, Kukler A, Heidt PJ, Valerio D: Long-term expression of human adenosine deaminase in rhesus monkeys transplanted with retrovirus-infected bone marrow cells. *Proc Natl Acad Sci USA* 89:7640, 1992
37. Schuening FG, Kawahara K, Miller AD, To R, Goehle S, Stewart D, Mullally K, Fisher L, Graham TC, Applebaum FR, Hackman R, Osborne WRA, Storb R: Retrovirus-mediated gene transduction into long-term repopulating marrow cells of dog. *Blood* 78:2568, 1991
38. Carter RF, Abrams-Ogg ACG, Dick JE, Kruth SA, Valli VE, Kamel-Reid S, Dube ID: Autologous transplantation of canine long-term marrow culture cells genetically marked by retroviral vectors. *Blood* 79:356, 1992